1 Data integration reveals key homeostatic mechanisms following low dose radiation exposure

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Abstract

The goal of this study was to define pathways regulated by low dose radiation to understand how biological systems respond to subtle perturbations in their environment and prioritize pathways for human health assessment. Using an *in vitro* 3-D human full thickness skin model, we have examined the temporal response of dermal and epidermal layers to 10 cGy X-ray using transcriptomic, proteomic, phosphoproteomic and metabolomic platforms. Bioinformatics analysis of each dataset independently revealed potential signaling mechanisms affected by low dose radiation, and integrating data shed additional insight into the mechanisms regulating low dose responses in human tissue. We examined direct interactions among datasets (top down approach) and defined several hubs as significant regulators, including transcription factors (YY1, MYC and CREB1), kinases (CDK2, PLK1) and a protease (MMP2). These data indicate a shift in response across time - with an increase in DNA repair, tissue remodeling and repression of cell proliferation acutely (24 – 72 hr). Pathway-based integration (bottom up approach) identified common molecular and pathway responses to low dose radiation, including oxidative stress, nitric oxide signaling and transcriptional regulation through the SP1 factor that would not have been identified by the individual data sets. Significant regulation of key downstream metabolites of nitrate stress were measured within these pathways. Among the features identified in our study, the regulation of MMP2 and SP1 were experimentally validated. Our results demonstrate the advantage of data integration to broadly define the pathways and networks that represent the mechanisms by which complex biological systems respond to perturbation.

Key Words: ionizing radiation, skin, oxidative stress, genomics, proteomics, metabolomics

Introduction

Mankind is exposed to low doses of natural and anthropogenic ionizing radiation every day. The growing use of ionizing radiation in industry, homeland security, research and particularly modern medicine continues to increase the potential for exposure. Understanding the biological effects of exposure to low dose / low dose rate ionizing radiation is therefore important for adequately protecting the population, and the environment, from any potentially detrimental effects of these increased radiation exposures (1).

Extrapolating from *in vitro* experimental systems where irradiated cells are grown in monolayers on the bottom of a plastic dish, *in vivo* experiments where animals are irradiated, or even epidemiological studies of exposed human cohorts are all useful but include uncertainties for determining mechanisms associated with low dose radiation exposures in humans. To overcome these challenges, our study utilizes a human *in vitro* 3-dimensional (3D) full thickness skin culture system which allows for mechanistic evaluation within heterogeneous cell types recapitulating *in vivo* human tissue response. The skin is the most radiation sensitive organ, however due to the ease with which damage or lesions can be identified and treated, it accounts for very low mortality as measured by cancer risk. Because of its sensitivity it makes an excellent model for looking at molecular level changes induced by low doses. Further, traditional radiation research has been a qualitative science in which parts of the process are studied individually by individual investigators. However, systems biology is enabling a transition from this qualitative science to a quantitative and ultimately predictive science. This transition is made possible by advances in technology that have created a wealth of new information revealing the enormous complexity underlying biological processes.

Our goal has been to develop a systems approach to understanding the biological effects of low dose (< 10 cGy), low dose rate (< 0.5 cGy/hr) exposure(s) to ionizing radiation. Specifically, how low dose / low dose rate exposures might modulate homeostasis in a 3D full thickness skin culture system *in vitro*. Human health effects following low dose radiation are

primarily extrapolated from high dose studies; however, quantitative and qualitative differences in cellular responses have been reported for these two dose ranges (2, 3). The low dose exposure parameters in our study mimic the dose limit for a full body spiral CT and the skin dose in the region of the steep dose gradient at the edge of the field in radiotherapy procedures (4). We have examined the temporal response of dermal and epidermal layers of the irradiated skin model system using transcriptomic, proteomic, phosphoproteomic and metabolomic strategies to generate a significant amount of heterogeneous data. While alterations in gene expression can directly result in alterations in protein abundance and activation of specific signaling pathways, a host of post-transcriptional and post-translational mechanisms also contribute to pathway activation. Therefore, by taking into account not only changes in gene transcription, but also alterations in protein abundance, protein post-translational modifications and the effects of altered protein activity (i.e. metabolic products), it is possible to identify novel signaling pathways not clearly elucidated by any single omic technology. To assess this, we performed systematic integration of multiple omic technologies using complementary approaches to identify the biological processes regulated at a systems-level by radiation exposure in skin. The use of complementary approaches allowed us to integrate data based on the unsupervised evaluation of multiple driving factors, including integration of data both at the pathway level and based on direct protein-protein interactions among the datasets. As a result, we have identified patterns of perturbed biological pathways that suggest persistent alterations in cellular / tissue homeostasis that in turn could alter sensitivity of skin following exposure to low dose radiation.

Materials and Methods

Experimental conditions. *In vitro* 3D full thickness human skin organotypic cultures were obtained from MatTek (EpiDermFT, MatTek Corporation, Ashland, MA). Tissue samples consist

of primary normal human epidermal keratinocytes and dermal fibroblasts derived from a single donor of neonatal foreskin. Fully developed 3D cultures are grown in a transwell insert at the air-liquid interface (**Figure 1**). Upon receipt, samples were randomized to exclude production specific alterations, placed in 6-well plates and fed daily with 2 ml of maintenance medium (MatTek Corporation, Ashland, MA). Maintenance Medium consists of Dulbecco's Modified Eagle's Medium containing 5 µg/ml gentamicin, 0.25 µg/ml amphotericin B, and phenol red without serum. Epidermal growth factor, insulin, hydrocortisone and other proprietary stimulators of epidermal differentiation are also present. Proprietary lipid precursors are used to enhance barrier formation. Cultures were maintained at 37°C, 95% humidity and 5% CO₂. Irradiations were performed using an X-rad 320 machine (300 kV, 1mA, Agfa NDT Pantak Seifert GmbH & Co.KG, Ahrensburg, Germany). Irradiated samples received a dose of 10 cGy delivered at a dose rate of 6.3 cGy/min. Consistent with the known physics and tract structure of ionizing radiation, the tissue construct will be fully penetrated and all cells exposed (see Figure 6.12(5)). Sham controls were placed in the irradiator but not exposed. Samples were collected at the time points indicated in **Table 1** for transcriptomics, proteomics, phosphoproteomics and metabolomics analysis. The global screens used for this paper have been published previously and readers are referred to those for additional experimental details (**Table 1**). The timepoints for collection of each data type were chosen based on preliminary data for low dose molecular changes in cells and based on relative differences in expected regulation of transcriptional, translational, post-translational and metabolic changes. Transcriptional analysis was performed on samples at 3, 24, and 72 hours post-irradiation to assess temporal progression of events from short- to longer-term after exposure. Metabolites were evaluated across an overlapping time course of 3, 24 and 48 hr after exposure. Phosphoproteomic changes that would be expected to occur shortly after exposure were measured after 1 hr. Preliminary proteomics data (unpublished) indicated significant changes in protein response 8 hours after exposure.

Global transcriptomics. At 3, 24, and 72 hours post-irradiation the dermis and epidermis were physically separated with forceps, which results in layers enriched for their constituent cell types, fibroblasts and keratinocytes, respectively. Details of the transcriptional microarray studies have been published previously (2) and only data processing is summarized here. Data were normalized using the Robust Multi-Array Analysis, and significantly regulated genes were identified with analysis of variance, Tukey's post-hoc test and Benjamini-Hochberg false discovery rate (FDR) statistics at $p < 0.05$ using the GeneSpring software (Agilent Technologies, Palo Alto, CA), as we have done previously (6).

Global proteomics. Quantitative proteomics were performed as described elsewhere (7). Basically, tissues were harvested at 8 hours after radiation exposure, and separated into cytoplasmic, nuclear and chromatin fractions in biological triplicate for statistical analysis. To prepare for iTRAQ labeling and MS analysis, proteins were reduced, alkylated and trypsin digested. Desalted peptide samples were labeled with 8-plex iTRAQ reagents (AB SCIEX, Foster City, CA) according to manufacturer's specifications and mixed in equal amounts. iTRAQ labeled peptides were separated using an online two dimensional liquid chromatography system (2D-LC) consisting of strong cation exchange (SCX) followed by reverse phase LC. MS data was searched using SEQUEST, and peptide identifications were filtered at 1×10^{-10} spectral probability using MSGF (8). Reporter ion counts were determined using MASiC (9). Results underwent significance testing using 1-way ANOVA at the peptide level and nested ANOVA at the protein level. Multiple testing error was accounted for using the Benjamini-Hochberg p-value correction. The resultant peptides and proteins with p-values less than 0.05 were deemed significant. Significant proteins for all cellular fractions were combined for data analysis and integration.

Phosphoproteomics. One hour post irradiation, tissue lysates were prepared by homogenization in 1 ml of freshly made 8 M urea in 50 mM ammonium bicarbonate (pH 8.0) containing a 1× concentration of phosphatase inhibitor (Thermo Fisher Scientific). Details of the sample preparation and phosphoproteomic studies have been published previously (10) and only data analysis is briefly summarized here. For each peptide we used a t-test to determine if the log ratio was significantly different than 0, which is equivalent to a paired t-test in that the difference between the raw log intensity values is equal to 0 (11).

Metabolomics. Tissues were collected at 3, 24 and 48 h post-irradiation with twelve replicates (n = 12) for each group and randomized across time point. Details of the global metabolomics analysis by GC-MS have been published previously (12) and only data processing is briefly summarized here. Metabolite identification was performed by searching chemically distinct features deconvoluted from the raw chromatogram against the Agilent Metabolomics GC-MS RTL (retention time locked) Library (Agilent Technologies, Palo Alto, CA). Metabolites were normalized using a statistical procedure for the analysis of proteomic normalization strategies (SPANS) that identifies the data scaling factor which introduces the least amount of bias into the dataset (13). The abundance values for all time points were normalized using a Rank Invariant approach – Median centering based on metabolite subsets from a Kruskal-Wallis p-value greater than 0.10. Comparative statistical analyses of time-matched sham control with irradiated samples were performed using a 2-sided Dunnett adjusted t-test to assess differences in average abundance, and a G-test to assess associations among factors due to the presence/absence of response (14).

Bioinformatics and data integration. All endpoints (genes, proteins, metabolites) that met significance criteria described above for each data type were included in downstream analysis and integration. Functional enrichment statistics were calculated for each dataset separately

using MetaCore v.6.20 (Thomson Reuters, Philadelphia, PA) to identify the most significant biological and metabolic processes affected by low dose irradiation in human skin tissue. The statistical scores were calculated using a hypergeometric distribution where the p-value represents the probability of a particular mapping arising by chance for experimental data compared to the background, which were different for each data type (15). The background for transcriptomics included all genes on the Affymetrix platform, whereas the background for proteomics, phosphoproteomics and metabolomics included all proteins or metabolites measured in each study. Data were integrated using Bioinformatics Resource Manager v2.3 (16) and based on known interactions from the MetaCore knowledgebase and relationships among the various high-throughput data types were statistically calculated based on the number of known connections between the datasets compared to the total number of connections in the knowledgebase. To identify major hubs or regulators within the integrated data, the Statistical Interactome tool (MetaCore) was used to measure the interconnectedness of genes in the experimental dataset relative to all known interactions in the knowledgebase. Statistical significance of over-connected interactions was calculated using a hypergeometric distribution. Networks were constructed for experimental data using an algorithm that identifies the shortest path (Metacore) to directly connect nodes in the dataset. Network visualizations were created in Cytoscape (17) utilizing the spring embedded layout with custom attributes to discriminate among data types, data sources and sub-networks.

Western Blot Analysis. Cells were processed for Western blot analysis, as previously described (18). Primary antibodies were used at a titer of 1:3000 and included P-SP1 (S¹⁰¹, Active Motif, Carlsbad, CA), SP1, cytokeratin 14 (K14) and vimentin (Santa Cruz Biotechnology, Dallas, TX) and vimentin. Horseradish peroxidase-conjugated secondary antibodies (Pierce, Rockford, IL) were used at 1:5000. Detection was carried out by ECL (SuperSignal West Femto,

Pierce, Rockford, IL) and quantification of luminescence was accomplished using a chemiluminescence imager (Lumilmager, Roche Molecular Biochemicals).

MMP2 Immunohistochemistry. EpiDerm^{TM} FT skin tissues collected at 3h, 24h 72h and 1 week after exposure were fixed in 4% paraformaldehyde in PBS, dehydrated, embedded in paraffin and cut into 5 µm sections (microtome, Leica RM 2155, Buffalo Grove, IL). Sections were deparaffinized in xylene and rehydrated via a series of alcohol rinses. Sections were processed for antigen retrieval by immersion in a citrate acid buffer (pH 6.0, 99°C) for 30 min which was cooled to room temperature over a 90 min period. The slides were rinsed in water and endogenous peroxidases were inactivated by incubation in 3% H₂O₂ for 10 min. The samples were washed 3x in PBS, permeabilized with 0.5% Triton X-100 (Sigma, St. Louis, MO) in PBS for 20 min, rinsed in PBS and nonspecific binding was blocked with 0.3% bovine serum albumin (Gibco, Auckland, New Zealand) in PBS for 1 hr. Sections were washed with PBS and incubated with 25 ug/ml Anti-human MMP-2 (R&D Systems, Minneapolis, MN) in blocking buffer over night at 4°C. All subsequent steps were carried out protected from light. Antibody binding was detected with 1 µg/ml donkey anti-mouse Alexa 488 (Invitrogen Corporation, Carlsbad, CA) in blocking buffer for 1 hr at room temperature. Samples were counterstained for 10 min with 300 nM 4',6-diamidino-2-phenylindole dilactate, DAPI (Invitrogen) in PBS. The slides were dehydrated and coverslipped with Cytoseal xyl (Thermo Fisher Scientific, Kalamazoo, MI). Individual tissue sections were imaged using a Nikon Eclipse TE300 fluorescence microscope with a Nikon Plan APO VC 60x/1.20 magnification water immersion objective. A Retiga 1300 cooled CCD camera (Qimaging) controlled by QCapture software was used to acquire the image. Equal exposure times were used to capture all tissue images. A minimum of 5 microscopic fields for each slide were randomly chosen, and the mean intensity of the epidermis was evaluated using MetaMorph (Molecular Devices, CA). Data are expressed as the

mean±SEM. T-tests were performed and p values < 0.05 were considered as statistically significant.

Results

High-throughput ‘multi-omics’ responses to low dose irradiation in skin. In this study we have utilized multiple approaches to integrate high-throughput ‘omics’ data to identify mechanisms associated with the homeostatic response of the skin to low dose irradiation. *In vitro* 3D reconstituted human skin tissue consisting of dermal fibroblast and epidermal keratinocyte layers was sham treated or irradiated with 10 cGy ionizing radiation for global transcriptomic, proteomic, phosphoproteomic and metabolomics analyses (**Table 1**). The manual separation of skin layers was evaluated by Western blot based on enrichment of proteins that are uniquely or predominantly detected in fibroblasts (vimentin) or keratinocytes (keratin 14, K14). As expected, vimentin and keratin 14 (K14) were highly enriched in layers containing fibroblasts (dermal) and keratinocytes (epidermal), respectively (**Figure 1B**). The individual datasets were each processed and analyzed separately to identify the significant genes, proteins or metabolites altered in skin tissue compared to sham control. Significant lists for each data type utilized for the integration are presented in **Supplemental File 1A-E**.

Top-down approach: Network-based integration of ‘omics’ data reveals significant interactions among datasets. For the top-down approach, we considered all direct interactions among the nodes (genes, proteins and metabolites) collectively across all datasets inferred from the MetaCore knowledgebase (**Figure 2**). Based on these interactions, we determined whether the connections between datasets were significantly greater than would be predicted by chance by hypergeometric distribution. The results, provided in **Table 2**, show that 3 of the datasets, global proteomics, global phosphoproteomics and the epidermal transcriptomics, are highly

interactive (ranging $p=1.756E-04$ to $p=1.991E-40$). To identify the upstream regulators driving transcript and protein changes predominately associated with the epidermal layer in response to low dose radiation, we determined the primary hubs within the integrated transcriptomic, proteomic and phosphoproteomic dataset. Hubs are defined as genes or proteins that are statistically over-connected to the experimental data based on known protein-protein interactions. When the number of connections between target genes and proteins in the dataset and the upstream regulatory hubs are greater than would be expected by chance, this indicates that the regulatory hubs are enriched under our experimental conditions and are important for regulating response to low dose radiation in skin. The most significant hubs include the transcription factors, YY1 ($p=3.68E-72$), MYC ($p=6.05E-68$) and CREB1 ($p=9.03E-52$), kinases CDK2 ($p=3.53E-59$) and PLK1 ($p=1.06E-44$) and protease MMP2 ($p=1.51E-47$). Both CREB1 and MMP2 were significantly regulated in our datasets by irradiation; CREB1 transcript was down regulated at 24 and 72 h post-irradiation and MMP2 protein was elevated at 8 h post-irradiation. Another significant hub, the kinase CDK1 ($p=3.81E-29$), was also significantly down regulated in the epidermal transcriptional dataset at 24 h post-irradiation.

We then created an integrative network model that included all the nodes with direct connections from the 3 datasets and included the 7 significant regulatory hubs identified above. The resulting top-down network, which incorporates 72% of the available data, is highly connected with regulatory patterns clearly distinguished in each sub-network through the significant hubs (**Figure 3**). To evaluate the changes that occur across multiple time points in response to low-dose exposure, we separated the integrated data sets into an early response (1-8 h post-exposure) which encompass all omic types; and a late response (24-72 h post-exposure) for which only the transcriptomic data is available. Of the transcripts and proteins that comprise the network, 50.2% are down-regulated during the early response, while 71.3% are down-regulated during the late response. This shift towards down-regulation of signaling at the later time points is significant ($p<0.0001$) by the Fisher Exact Test. To visualize the nodes

involved in this shift during the late response, we colored the nodes in **Figure 3** based on time point and direction of change. All nodes down-regulated during the late response are dark blue, whereas nodes up-regulated during the late response are orange and nodes regulated during the early response are gray. With the data clearly identified by early and late response in **Figure 3**, we can now observe trends in gene regulation specifically for each sub-network contributing to a better understanding of mechanism of action. For example, most of the nodes in the CREB1 sub-network are significantly altered by radiation exposure after 24 hours during the later response period (67% during late response compared to 33% during early response). In contrast, over 87% of the nodes in the MMP2 network are significantly changed early (1-8 hrs) after exposure with only 13% altered during the late response period. While nodes in the other sub-networks are regulated equivalently (~50%) during the early and late responses, they are predominantly down-regulated during the late response (73-90% depending on network) supporting the trend observed in the overall integrated dataset.

We have further identified the significantly enriched ($p < 0.0001$) biological processes for each sub-network to understand the mechanisms regulated by low-dose irradiation within the 'top down' integrated network (**Figure 3**, text annotation). These processes, when interpreted within the context of observed temporal changes, provide a more comprehensive understanding of changes associated with the epidermis in response to low dose radiation exposure. In particular, we observed altered genes and proteins from cell adhesion, cell matrix interaction and endoplasmic reticulum stress (ER) stress response (protein folding) pathways at early time points that were associated with an increase in MMP2 protein. We have experimentally confirmed the increase in MMP2 protein levels in the skin model at 10 cGy as early as 3 hr post-irradiation (**Figure 4**). Further, by comparing changes in MMP2 across dose and time, we see that it is elevated with treatment and this change persists up to a week post-irradiation. When we evaluate the significant processes regulated during the early response (1-8 hr) and late response (24-72 hr) periods overall (**Supplemental Figure 1**), we see that these changes in cell

structure and communication are up-regulated in the early response along with other cytoskeletal, tissue remodeling and cell adhesion processes. Alternatively, processes associated with cell cycle and double-strand break DNA repair are predominately down-regulated during the late response and are represented within the MYC, CREB1 and CDK1/2 signaling networks. One biological process for heat shock stress response to unfolded proteins met significance criteria during both time periods; although it was significantly increased during the early response and decreased during the late response (**Supplemental Figure 1**). These data indicate a shift in response across time in the epidermis in which there is an early increase in tissue remodeling during the first 8 hr post-irradiation and then repression of cell proliferation and DNA repair at 24-72 hr.

Bottom-up approach: Pathway-based integration of ‘omics’ data reveals importance of Sp1-mediated oxidative stress in homeostatic response. For the bottom-up approach, we first analyzed each dataset individually for significantly enriched biological and metabolic processes to identify those that are common to each data type and then created an integrated network for the transcripts, proteins and metabolites associated with related processes common across all datasets (**Figure 2**). The significantly enriched biological and metabolic processes regulated in skin by exposure to low dose radiation are presented for each data type in **Figure 5**. While the processes significant for the transcripts in the epidermis and the dermis had the most overlap among the datasets, they were still distinct from each other indicating that the two layers respond differently to low levels of radiation. Overall, processes associated with cell cycle, DNA damage and DNA repair were more significant for the epidermal layer compared to the dermal layer. The most significant biological processes regulated by low dose radiation in the dermal layer were associated with apoptosis, stress response, nitric oxide signaling, regulation of epithelial-to-mesenchymal transition, cell proliferation and TGF-beta signal transduction pathways.

While there was little direct overlap in the biological and metabolic processes significantly enriched across the transcriptomic, proteomic, phosphoproteomic and metabolomic datasets (**Figure 5**), we observed that some of the most significant pathways regulated within each data type were associated with oxidative and nitrative stress response after low dose radiation exposure in skin (summarized in **Supplemental Figure 2**). For example, the most significant process enriched for the global metabolomics data was nitric oxide biosynthesis and multiple processes for nitric oxide signaling and reactive oxygen species (ROS) were enriched in the dermal transcriptomics. Other processes related to ER stress response and phosphatidylinositol-4,5-diphosphate (PIP2) signaling, which are consequences of ROS and nitric oxide signaling, were also significant across the epidermal transcriptomics, dermal transcriptomics, proteomics and phosphoproteomics (**Supplemental Figure 2**). In order to visualize these processes combined, the associated transcripts, proteins and metabolites were integrated based on known interactions from the MetaCore knowledgebase (**Figure 6A**). An enlargement of the metabolic process for nitric oxide biosynthesis is shown in **Figure 6B** with the metabolite changes detected in skin after low dose radiation exposure included in the network. These metabolites were significantly altered in skin at 10 cGy compared to sham control such that there was a decrease in urea levels as early as 24 hr post-irradiation (**Figure 7**). After 48 hrs post-irradiation, there were significant changes in other metabolites measured in this pathway, including a decrease in L-lysine and increases in L-ornithine, citrulline and N,N-dimethylarginine levels. This suggests a shift away from urea synthesis and towards nitric oxide synthesis since both processes compete for arginine as a substrate. The most significant transcription factor regulating the nodes in the combined network is SP1 ($p=4.6E-25$), which was added to the network in Figure 4A even though it was not measured in any of the datasets. We were able to validate phosphorylation of SP1 in the skin model 24 hr post-irradiation with 10 cGy exposure that was 3-fold greater than sham control, by Western blot (**Figure 8**). Phosphorylation of SP1 continued to be elevated 28-fold compared to sham after 200 cGy

irradiation. These data suggest that signaling in the integrated oxidative stress network is mediated, at least in part, through phosphorylation of SP1, and the metabolomics data indicate that these changes persist up to 48 hr post exposure.

Discussion

Systems biology approaches for data integration and interpretation of multiple high-throughput omic datasets are necessary for understanding complex biological systems. In this study we have measured the response of the keratinocytes in the epidermis and fibroblasts in the dermis to low dose ionizing radiation exposure over the period of 1 – 72 hrs post-irradiation using global transcriptomics, proteomics, phosphoproteomics and metabolomics. Our goal was to determine the key signaling pathways of altered cellular and tissue homeostasis after low dose exposure based on integrated networks of genes, proteins and metabolites significantly changed across time in our model system. We have previously found that signaling networks derived from integrated data are more highly connected and more likely to accurately reconstruct cellular responses (19). Therefore, we propose that the use of integrated data provides both a more complete understanding and a higher confidence in the identified mechanisms associated with exposure and disease states. We describe two complementary approaches for developing integrative networks in order to identify the most significant signaling events and the key upstream regulators and regulatory mechanisms in skin after low dose radiation exposure (**Figure 2**). The utilization of multiple methods for data integration allowed us to approach the complex task of analyzing data from several global omic technologies in a comprehensive and systematic manner. While both approaches are equally valid, they provided unique (yet complementary) views of the mechanisms regulated in skin during the hours post exposure at low doses. Since the methods chosen for reducing the complexity of multi-omic data analysis strongly influence the final interpretation and biological understanding; the use of multiple approaches for data reduction and analysis are increasingly utilized to provide a more

comprehensive and less biased perspective of systems level changes (20). We were able to confirm regulation of proteins that were computationally predicted to be significant regulators of radiation-induced gene signaling through both approaches.

Matrix Regulation. In the first approach, which we have termed ‘top-down’, we considered all of the available data and developed the integrative network using the datasets that were most significantly connected based on known protein-protein interactions. We then identified the primary regulatory hubs associated with the sub-networks and the significant biological processes regulated in each sub-network (**Figure 3**) or regulated across time (**Supplemental Figure 1**). This process provided us with a better view of the radiation-induced changes occurring in skin as a whole following low dose exposure. In particular, we found that the proteomic and phosphoproteomic changes measured were most significantly associated with transcriptional changes in the epidermis. Since global proteomics preferentially detects the more highly abundant structural proteins, these data suggest that the significant structural changes occurring in skin in response to low dose irradiation were primarily occurring in keratinocytes in the epidermal layer. Specifically, we observed a significant increase in genes and proteins associated with tissue remodeling, cell structure and cell communication processes early (8 hr) after exposure to low dose irradiation in the epidermis mediated by MMP2, which was significantly elevated itself in addition to MMP1 and other extracellular matrix (ECM) proteins, tenascin, fibromodulin, lumican, nidogen and TIMP1. While elevation of MMP1 and MMP2 are associated with invasive and metastatic properties of tumors, they are also important during normal tissue remodeling, wound healing and inflammation associated with tissue damage. Epidermal wound repair is a complex process coordinated by matrix metalloproteinases MMP1 and MMP2, among others, and involves stages of hemostasis, inflammation, migration, proliferation and remodeling (reviewed in (21)). MMP2 has previously been identified as a marker associated with an early response (4 hr) to low dose irradiation in mouse skin (22). We observed a consistent increase in MMP2 protein abundance by

immunohistochemistry at 3 hr, 24 hr, 72 hr and 1 wk post-irradiation with 10 cGy indicating that ECM-associated changes were persistent after exposure.

Cell Cycle Regulation and DNA Damage. In addition to the early changes associated with tissue remodeling measured in the epidermis, we also observed an overall decrease in genes and proteins associated with cell cycle and DNA damage mechanisms during the late response period (24-72 hr) post-irradiation. These signaling processes were mediated by the regulators CREB1, MYC and CDK1/CDK2/PLK1 in the network. We have previously observed a decrease in epidermal cell proliferation 24-72 hr after 10 cGy irradiation as measured by reduced incorporation of EdU into proliferating cells and down regulation of the proliferative marker MKi67 (2). Our current data show that several genes and proteins associated with cell cycle and DNA damage checkpoint regulation were significantly decreased in the epidermis, including CDC2, CDC7, CCND1, TOP1, TOP2, TOPBP, MCM3 and ATR. Many of the genes are associated with p53 response and suggest possible cell cycle arrest 24-72 hrs after exposure to low dose irradiation. ATR (ataxia telangiectasia mutated–Rad3-related protein) participates in the activation of cell-cycle checkpoints and repair induced by DNA damage, its loss in cells has previously been found to potentiate effects of DNA-damaging chemotherapeutic drugs on cancer cell survival associated with increased sensitivity to radiation-mediated DNA damage (23, 24). Low dose hyper-radiosensitivity has been measured in over 75% of the cancer and normal cell lines tested, including in human and rodent skin models (25). Our observation that low dose radiation exposure in skin tissue results in regulation of double strand break DNA damage and repair pathways suggests that a similar paradigm of low-dose hypersensitivity may be relevant in our model.

Oxidative and Nitrate Stress in Wound Healing. We have further utilized a second network integration approach in which we identified the most significant biological processes altered by each data type individually and then created an integrated network for genes and proteins associated with oxidative and nitrate stress response, which was common among

most data types. While these processes were significant in datasets associated with the epidermis, dermis and whole skin, they were most significant in the dermal fibroblasts, suggesting that this layer located deeper in the skin is more sensitive to irradiation-induced oxidative stress. Other studies have reported a similar response in reconstructed skin after exposure to non-extreme ultraviolet irradiation in which the dermal layer was more susceptible to transcriptional changes associated with measured oxidative stress (26). In our study, we identified the transcription factor, SP1, as the most significant upstream regulator of the integrated oxidative stress network. Since the identification of SP1 as a significant regulator was calculated based on its connection to all genes, proteins and metabolites within the integrated network, we likely would not have observed its significant enrichment using the pathways for each data type independently providing an example of the added benefit of utilizing integrated datasets for understanding complex mechanisms. SP1 has previously been identified as a redox-regulated transcriptional activator in other models (27-29) and recent evidence describes a role for SP1 during DNA damage repair (30-32). Human fibroblasts exposed to ionizing radiation at higher doses of 10-40 Gy resulted in phosphorylation of SP1 that localizes with the double-strand DNA damage marker γ H2AX at double strand break sites and promotes repair (30). The consequence of these changes after low doses of radiation that do not result in measurable DNA damage is unknown, but suggests a role for SP1 in regulating radiation-induced stress response. Increased phosphorylation of SP1 has previously been associated with cell cycle arrest, apoptosis and radiosensitivity after 450-600 cGy irradiation in malignant melanoma cells (32). In our SP1 network, several redox-related genes and proteins were elevated after low dose radiation exposure, including mitochondrial superoxide dismutase (SOD2), several heat shock proteins and mitochondrial apoptosis (BAK, BAX, CASP3 and cytochrome c).

In addition to the matrix metalloproteinase response (described above) regulated in response to epidermal wound repair, wound healing and repair in response to radiation

exposure can result in elevated oxidative and nitrative stress and increased levels of SOD2 and expression of arginase-1 (ARG1) (33). In response to low dose irradiation in skin, we also observed elevated ARG1, which converts L-arginine to L-ornithine and urea in the urea cycle. L-arginine is the primary substrate for synthesis of nitric oxide (NO). Nitric oxide synthase (NOS), which competes with arginase for use of arginine, produces NO and citrulline. We observed significant alterations in nitric oxide signaling and biosynthesis in our study, resulting in increased L-ornithine and citrulline suggesting activation of both of these reactions. However, because NOS and arginase compete for arginine, it has been suggested that elevation of arginase is protective against formation of NO in cells since it reduces the amount of L-arginine available for NO production (34). We also observed increased levels of the metabolite N,N-dimethylarginine and the enzyme dimethylarginase (DDAH1) after 10 cGy irradiation in skin. The role of both of these is to block NO synthesis, while its elevation in transgenic animals is associated with increase NOS activity. The detection of these metabolites in our study and integration with the mechanisms measured at the transcriptional and proteomic levels provide additional insight into the role of nitrative stress and nitric oxide biosynthesis in response to low dose radiation in skin, which persists up to 48hr post exposure.

Products of oxidative stress, including ROS, RNS and, in particular, NO, have recently been identified as initiators of radiation-induced bystander and adaptive responses (35, 36). The tissue level response to restore homeostasis subsequent to low dose radiation exposure can result in non-targeted effects, including both adaptive and bystander effects in cells and tissues. While the consequences of these events, such as sensitivity to subsequent exposures, are outside the scope of this study, the mechanisms we have identified through global integration of systems level data after low dose radiation exposure in skin tissue indicate that NO-mediated signaling during an initial stress response may prime cells for subsequent radioadaptive response, accumulation of inducible NOS and activation of p53 pathways. Some of the consequences of ROS/RNS-mediated signaling reported after initial low dose radiation

exposure are repression of cell proliferation and double-strand break repair pathways, which have been proposed as part of p53-dependent adaptive mechanisms (37, 38). In addition to the significant alteration of NO biosynthesis and ROS pathways measured in our study, we also observed repression of cell proliferation and double-strand break DNA repair pathways at 24-47 hours post-irradiation in skin supporting a role for these mechanisms during homeostasis. Overall, our data suggest that exposure to low doses of radiation produces an altered homeostatic state within skin, involving both temporal and location-specific changes in matrix regulation, ROS/RNS stress response pathways, cell proliferation and DNA repair, which could potentially alter sensitivity to a subsequent challenge or environmental change.

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Figure Legends

Figure 1. Human skin as a model system. *In vitro* 3D reconstituted human skin tissue (EpiDermFT, MatTek) consisting of dermal fibroblast and epidermal keratinocyte layers was sham treated or irradiated with 10 cGy ionizing radiation. Genes, proteins and metabolites were measured by various methods over the course of 1 to 72 hrs post-irradiation. The layers were separated into epidermal and dermal sections for global mRNA transcriptomic (T) analysis at 3, 24 and 72 hr post-irradiation. Full skin tissue was utilized for global proteomic (P), phosphoproteomic (Ph) and metabolomic (M) analyses at time points ranging from 1 to 48 h post-irradiation (**Table 1**). After separation of skin layers, enrichment of keratin 14 (K14) and vimentin proteins was measured by Western blot in the epidermal keratinocyte and dermal fibroblast layers, respectively, compared to actin as a loading control.

Figure 2. Comparison of complementary data integration approaches. Schematic describes ‘top-down’ and ‘bottom-up’ approaches utilized for integration of global transcriptomic, proteomic, phosphoproteomic and metabolomics datasets to identify the most significant signaling events and key regulatory mechanisms in skin after low dose radiation exposure.

Figure 3. Integrated network of the most highly connected high-throughput datasets measured in skin after low dose irradiation (top-down approach). Interactions among the global proteomics, phosphoproteomics and epidermal transcriptomics datasets were predicted to be highly overconnected (**Table 2**) compared to that expected by chance. The network containing nodes from these datasets is significantly enriched for signaling through CREB1 (blue), MYC (green), YY1 (purple), MMP2 (orange) and kinases CDK1, CDK2 and PLK (red) and the edges are colored for each sub-network based on its central hub. The significant biological processes ($p < 0.0001$) regulated by genes and proteins in each sub-network is shown

(based on color). Nodes are shaped based on data type for transcripts (circle), proteins (triangle), phosphoprotein (diamond) or from multiple data types (square). Nodes are colored based on the data source with the nodes regulated during the early response (1-8 h post-irradiation) in gray, nodes up-regulated during the late response (24-72 h post-irradiation) in orange and nodes down-regulated in the late response in blue.

Figure 4. Relative fluorescence intensity of epidermal MMP2 in skin post-irradiation.

MMP2 protein expression was measured by immunohistochemistry as a function of time and dose post exposure to X-rays. Data are expressed as the mean $\pm$ SEM (N=5 replicates). Significance by T-test is indicated in comparison to time-matched sham control (* $p < 0.05$, ** $p < 0.01$).

Figure 5. Significant enrichment of biological and metabolic processes after 10 cGy

irradiation. Heatmap shows top 10 significantly ($p < 0.05$) enriched biological and metabolic processes for each dataset in human skin model after 10 cGy irradiation. Stress response pathways, including heat shock and oxidative/ nitrative stress, are significant across most of the datasets, including metabolomic data. Cell cycle, DNA damage and apoptotic responses are primarily regulated at the transcriptional level, while cytoskeleton and cell adhesion processes are regulated at the protein level. Functional enrichment statistics were calculated in Metacore (GeneGo, St. Joseph, MI) using a hypergeometric distribution where the p-value represents the probability of a particular mapping arising by chance for experimental data compared to the background, which included all entities measured or detected for each data type. Epi = Epidermis; Der = Dermis; FT = Full tissue

Figure 6. Integrated network of all transcripts, proteins, phosphoproteins and metabolites associated with oxidative stress response pathways in skin after low dose

irradiation (bottom-up approach). A) The combined network from all datasets is mediated through SP1 signaling as determined by transcription factor enrichment analysis ($p=4.6E-25$). Nodes are shaped based on data type for transcripts (circle), proteins (triangle), metabolites (square) or both transcript/protein (diamond). Nodes are colored based on the data source for dermal transcriptomics (blue), epidermal transcriptomics (red), phosphoproteomics (orange), global proteomics (yellow), metabolomics (gray), multiple data sources (green). **B)** Enlargement of the network shows details of the metabolomics endpoints involved in nitric oxide biosynthesis. Numbers refer to the following metabolic reactions:

¹ $H_2O + L\text{-Arginine} = L\text{-Ornithine} + \text{Urea}$

² $NAD(P)H + O_2 + L\text{-Arginine} = NO + L\text{-Citrulline} + NADP(+) + H_2O$

³ $N,N\text{-Dimethylarginine} + H_2O = \text{Dimethylamine} + L\text{-Citrulline}$

⁴ mitochondrial matrix $H(+)$ + mitochondrial matrix L-Citrulline + cytosol L-Ornithine = cytosol

$H(+)$ + cytosol L-Citrulline + mitochondrial matrix L-Ornithine

⁵ mitochondrial matrix L-Lysine + cytosol L-Ornithine = cytosol L-Lysine + mitochondrial matrix

L-Ornithine

Figure 7. Metabolites in oxidative stress network. All metabolites measure in the nitric oxide biosynthetic pathway were significant ($p<0.05$) after 10 cGy irradiation by G-test based on the presence or absence of the metabolite measured in irradiated skin samples compared to sham controls. The heatmap visualization represents the counts (or number of biological replicates out of 12 total) in which the metabolite was detected 48 h after irradiation with 0 (sham), 3 or 10 cGy. *Urea was significant 24 h post-irradiation.

Figure 8. Phosphorylation of Sp1 by low dose irradiation. Western blots of phosphorylated SP1 (P-SP1) and SP1 protein after irradiation of the human skin model with 10 and 200 cGy compared to sham control. The increase in SP1 phosphorylation with irradiation is presented as

729 the ratio of P-SP1 to SP1 for sham control, 10 cGy and 200 cGy samples and fold-change is
730 normalized to sham controls.

Table 1. Summary of high-throughput data collected from *in vitro* reconstituted human skin tissue model for integration

Data Type	Sample	Dose	Timepoints
Transcriptomics ^a	Epidermis	10 cGy	3, 24, 72 hr
Transcriptomics ^a	Dermis	10 cGy	3, 24, 72 hr
Global proteomics	Full Tissue (chromatin/nuclear/cytoplasm)	10 cGy	8 hr
Phosphoproteomics ^b	Full Tissue	10 cGy	1 hr
Metabolomics ^c	Full Tissue	10 cGy	3, 24, 48 hr

^a (2)

^b (10)

^c (12)

Table 2. Significant interactions measured among high-throughput datasets

Dataset 1	Dataset 2	p-value ^a	z-score ^b
Phosphoproteomics (FT)	Global proteomics (FT)	1.991E-40	14.99
Transcripts (Epi)	Global proteomics (FT)	6.881E-17	8.502
Transcripts (Epi)	Phosphoproteomics (FT)	1.756E-04	3.658
Transcripts (Epi)	Transcripts (Der)	6.079E-03	2.52
Transcripts (Der)	Global proteomics (FT)	1.847E-02	2.097
Transcripts (Der)	Phosphoproteomics (FT)	3.454E-01	-0.4072

FT = Full tissue; Epi = Epidermis; Der = Dermis

^a The p-value is the probability that the number of connections between datasets are greater/lesser than would be expected compared to the total number of connections in the MetaCore (Thomson Reuters) knowledgebase.

^b The z-score is calculated as ((Actual-Expected)/sqrt(variance)).

Figure

Figure 2

Top-Down Approach

Bottom-Up Approach

Figure 3

Figure 4

Figure 5

Figure 6A

Figure 6B

Figure 7

Figure 8